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Secondary Metabolite Profiling and In Vitro Bioefficacy of *Retama raetam* Extracts Using Bacterial Test Systems.

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التوصيف الكيميائي للمستقلبات الثانوية وتقييم الفعالية الحيوية لمستخلصات نبات الرتم (*Retama raetam*) في المختبر باستخدام أنظمة اختبار بكتيرية

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Abstract:
The increasing global concern regarding reduced effectiveness of conventional antimicrobial agents has intensified interest in identifying alternative bioactive resources of natural origin. This study evaluated the phytochemical profile and in vitro bioefficacy of *Retama raetam* crude extracts using selected bacterial test systems, namely *Escherichia coli, Klebsiella spp., Pseudomonas aeruginosa*, and *Staphylococcus aureus*. Aerial parts of the plant were collected from Al-Zawiya, Libya, shade-dried, and extracted using ethanol, methanol, and distilled water through cold maceration. Extraction yield varied according to solvent polarity, with ethanol producing the highest yield. Qualitative phytochemical screening revealed the presence of alkaloids, flavonoids, tannins, terpenoids, and saponins, with a comparatively higher abundance of bioactive constituents detected in the ethanolic extract. Biological response was assessed using the agar well diffusion assay against standardized inocula (0.5 McFarland). The ethanolic extract demonstrated pronounced inhibitory activity, particularly against P. aeruginosa (40 ± 1.2 mm), while moderate inhibition was observed against *S. aureus* and *E. coli*. No detectable inhibition was recorded against *Klebsiella spp*. Statistical analysis using one-way ANOVA confirmed significant differences among extracts ($p \leq 0.05$). The observed bioactivity may be attributed to the synergistic action of phenolic compounds and alkaloids capable of influencing bacterial membrane integrity and metabolic pathways. These findings position *R. raetam* as a promising reservoir of biologically active secondary metabolites and support further quantitative, mechanistic, and minimum inhibitory concentration investigations to clarify its functional potential.

Keywords: *Retama raetam*, phytochemical screening, antibacterial activity, secondary metabolites, antimicrobial resistance.

الملخص
إن التزايد العالمي في القلق المرتبط بتراجع فعالية العوامل المضادة للميكروبات التقليدية قد عزز الاهتمام بالبحث عن مصادر حيوية بديلة ذات أصل طبيعي. هدفت هذه الدراسة إلى تقييم النمط الكيميائي النباتي والفعالية الحيوية في المختبر لمستخلصات نبات الرتم (*Retama raetam*) الخام باستخدام نظم اختبار بكتيرية مختارة، وهي: *Escherichia coli* و*Klebsiella spp*. و*Pseudomonas aeruginosa* و*Staphylococcus aureus*. تم جمع الأجزاء الهوائية للنبات من مدينة الزاوية، ليبيا، وجُففت في الظل، ثم استُخلصت باستخدام الإيثانول والميثانول والماء المقطر بطريقة النقع البارد. اختلفت نسبة الاستخلاص تبعًا لقطبية المذيب، حيث حقق الإيثانول أعلى مردود استخلاصي. كشف الفحص الكيميائي النباتي النوعي عن وجود القلويدات والفلافونويدات والتانينات والتربينويدات والصابونينات، مع تسجيل وفرة أعلى نسبيًا

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من المركبات الحيوية الفعالة في المستخلص الإيثانولي. تم تقييم الاستجابة الحيوية باستخدام اختبار الانتشار في الآجار بطريقة الآبار ضد لقاحات بكتيرية قياسية بتركيز 0.5 ماكفارلاند. وأظهر المستخلص الإيثانولي نشاطًا تثبيطيًا ملحوظًا، لا سيما ضد *P. aeruginosa* (40 ± 1.2 ملم)، في حين لوحظ تثبيط متوسط ضد *S. aureus* و*E. coli*، ولم يُسجل أي تثبيط قابل للرصد ضد *Klebsiella spp*. أكد التحليل الإحصائي باستخدام تحليل التباين الأحادي وجود فروق معنوية بين المستخلصات عند مستوى دلالة ($p \leq 0.05$). ويُحتمل أن تُعزى الفعالية الحيوية الملحوظة إلى التأثير التآزري للمركبات الفينولية والقلويدات القادرة على التأثير في سلامة الغشاء البكتيري والمسارات الأيضية. وتشير هذه النتائج إلى أن نبات الرتم يمثل مخزونًا واعدًا من المستقلبات الثانوية ذات النشاط الحيوي، وتدعم إجراء دراسات كمية وآلية إضافية، بما في ذلك تحديد التركيز المثبط الأدنى، لتوضيح إمكاناته الوظيفية بصورة أدق.

الكلمات المفتاحية :الرتم (*Retama raetam*)، الفحص الكيميائي النباتي، النشاط المضاد للبكتيريا ،المستقلبات الثانوية، مقاومة المضادات الحيوية.

1-Introduction

Medicinal plants represent one of the most enduring and chemically diverse reservoirs of bioactive secondary metabolites. Unlike primary metabolites, which are directly involved in growth and development, secondary metabolites function largely in ecological defense and environmental adaptation. Among the most studied classes are alkaloids, flavonoids, tannins, terpenoids, and saponins, many of which demonstrate pronounced antimicrobial activity (Cowan, 1999; Daglia, 2012). These compounds exert antibacterial effects through multiple mechanisms, including disruption of cytoplasmic membrane integrity, inhibition of essential enzymes, interference with nucleic acid synthesis, metal ion chelation, and induction of oxidative stress within microbial cells. The structural diversity of plant-derived molecules provides a biochemical advantage in targeting resistant pathogens, often through multi-site modes of action that reduce the likelihood of rapid resistance development.

In arid and semi-arid ecosystems, plants are subjected to intense environmental stressors such as high temperature, ultraviolet radiation, water scarcity, and nutrient limitation. These stress conditions frequently stimulate enhanced biosynthesis of protective secondary metabolites. *Retama raetam* (Forssk.) Webb & Berthel., belonging to the family Fabaceae, is a xerophytic shrub widely distributed across North Africa and parts of the Middle East. It thrives in sandy and desert habitats, where its ecological resilience is associated with adaptive metabolic pathways. Previous phytochemical investigations have identified quinolizidine alkaloids, flavonoids, and phenolic constituents within *R. raetam*, suggesting potential pharmacological relevance (Hammouche-Mokrane,2017). Traditional uses of the plant in local ethnomedicine for inflammatory and infectious conditions further support its therapeutic potential.

The renewed scientific interest in plant-derived antimicrobials is largely driven by the accelerating global crisis of antimicrobial resistance (AMR). Opportunistic pathogens such as *Pseudomonas aeruginosa, Klebsiella pneumoniae, Escherichia coli,* and *Staphylococcus aureus* are increasingly associated with hospital- and community-acquired infections exhibiting multidrug resistance profiles. According to the World Health Organization (WHO, 2023), antimicrobial resistance threatens the effectiveness of existing therapeutic regimens and imposes substantial clinical and economic burdens worldwide. Tacconelli et al. (2018) emphasized the urgent

need for novel antimicrobial agents, particularly those targeting priority pathogens characterized by intrinsic and acquired resistance mechanisms, including efflux pumps, enzymatic drug inactivation, biofilm formation, and capsule-mediated protection.

P. aeruginosa, for instance, possesses a highly impermeable outer membrane and multiple efflux systems that reduce intracellular drug accumulation, rendering many conventional antibiotics ineffective. Similarly, *K. pneumoniae* exhibits a polysaccharide capsule that enhances virulence and protects against host immune responses as well as antimicrobial agents. These adaptive features complicate treatment strategies and underscore the necessity of identifying alternative compounds with novel modes of action. Natural products derived from plants offer a promising avenue, particularly when their complex phytochemical matrices may exert synergistic antibacterial effects.

Although several studies have examined the phytochemistry of *R. raetam*, comprehensive correlations between its qualitative phytochemical composition and antibacterial activity against clinically relevant pathogens remain limited, especially in the Libyan context. Environmental factors such as soil composition, climate, and geographical location can influence secondary metabolite production, thereby affecting biological activity. Therefore, region-specific investigations are essential to evaluate the therapeutic potential of locally sourced plant material.

The present study was designed to investigate the phytochemical profile of *R. raetam* crude extracts prepared using solvents of different polarities and to assess their in vitro antibacterial activity against selected clinical isolates of E. *coli*, *Klebsiella spp.*, *P. aeruginosa*, and *S. aureus*. By integrating phytochemical screening with antimicrobial susceptibility assays, this work aims to contribute to the growing body of evidence supporting desert plants as valuable sources of bioactive compounds and to provide a scientific basis for future quantitative, mechanistic, and pharmacological investigations.

2. Materials and Methods

2.1 Plant Material

Aerial parts of *Retama raetam* were collected during spring 2019 from Al-Sayeda Zainab region, Al-Zawiya, Libya. Plant identification was confirmed using standard taxonomic keys. Samples were washed, shade-dried (25 ± 2°C), ground, and stored in dark containers.

2.2 Preparation of Extracts

Ninety grams of powdered plant material were macerated in 200 mL of ethanol (96%), methanol, or distilled water for 72 h at room temperature with intermittent shaking (Azwanida, 2015). Extracts were filtered and concentrated using a rotary evaporator at 40°C. Crude extracts were stored at 4°C.

Extraction yield (%) was calculated as:

Extraction yield (%) = (Weight of dried extract / Weight of plant powder) × 100

2.3 Phytochemical Screening

Preliminary qualitative phytochemical analysis of *Retama raetam* crude extracts was performed to detect major classes of secondary metabolites, following standard protocols described by Harborne (1998) and Trease & Evans (2009), with minor modifications to improve sensitivity and reproducibility.

Alkaloids were detected using Dragendorff's and Mayer's reagents. A few drops of Dragendorff's reagent were added to 2 mL of extract solution acidified with 1% HCl. The formation of an orange-brown precipitate indicated the presence of alkaloids. Confirmation was performed using Mayer's reagent, where a creamy white precipitate suggested positive reaction.

Flavonoids were identified using the Shinoda test. Briefly, small magnesium turnings were added to the extract followed by concentrated hydrochloric acid. The appearance of a pink to reddish coloration indicated the presence of flavonoids. Additionally, alkaline reagent testing was performed by adding 10% NaOH solution; the formation of intense yellow color that became colorless upon addition of dilute acid confirmed flavonoid presence.

Tannins were detected using the ferric chloride test. A few drops of 5% $FeCl_3$ solution were added to the extract. The formation of a blue-black or greenish coloration indicated hydrolysable or condensed tannins, respectively.

Saponins were evaluated using the froth test. The extract was vigorously shaken with distilled water in a graduated cylinder for 30 seconds and allowed to stand for 15 minutes. The formation of a stable persistent froth (≥1 cm height) indicated the presence of saponins.

Terpenoids were detected using the Salkowski test. The extract was mixed with chloroform followed by careful addition of concentrated sulfuric acid along the test tube wall. A reddish-brown interface indicated the presence of terpenoids.

Glycosides were screened using Keller–Killiani test. The extract was treated with glacial acetic acid containing traces of ferric chloride, followed by addition of concentrated sulfuric acid. The appearance of a brown ring at the interface suggested the presence of cardiac glycosides.

All tests were performed in triplicate to ensure consistency of observations. Results were recorded qualitatively as absent (–), weak (+), moderate (++), or strong (+++), based on visual intensity of reaction.

2.4 Bacterial Strains

Clinical isolates of *E. coli*, *Klebsiella spp.*, *P. aeruginosa*, and *S. aureus* were obtained from the Microbiology Laboratory, Faculty of Science, University of Tripoli. Inocula were standardized to 0.5 McFarland (CLSI, 2023).

2.5 Antibacterial Assay

The antibacterial activity of *Retama raetam* crude extracts was evaluated using the agar well diffusion method, a standardized qualitative screening technique widely employed for preliminary assessment of antimicrobial potential (Bauer et al., 1966; Balouiri et al., 2016). The procedure was conducted in accordance with general

guidelines for antimicrobial susceptibility testing to ensure methodological reliability (CLSI, 2023).

Fresh overnight bacterial cultures were adjusted to 0.5 McFarland turbidity standard, corresponding to approximately 1×10^8 CFU/mL, to achieve uniform inoculum density. The standardized suspensions were evenly spread onto Mueller–Hinton agar plates using sterile cotton swabs to obtain confluent bacterial lawns. The plates were allowed to dry for 10–15 minutes under aseptic conditions prior to well preparation.

Sterile cork borers were used to punch wells of 8 mm diameter into the agar. Each well was filled with 50 µL of crude extract solution under sterile conditions. Care was taken to prevent overflow and ensure uniform diffusion within the agar matrix. Plates were left undisturbed at room temperature for approximately 30 minutes to allow initial radial diffusion before incubation.

The inoculated plates were incubated aerobically at 37°C for 18–24 hours. After incubation, the diameters of inhibition zones were measured in millimeters (mm) using a calibrated digital caliper. The well diameter was included in the total measurement. All experiments were performed in triplicate, and results were expressed as mean ± standard deviation.

Solvent-only wells served as negative controls to exclude antimicrobial effects attributable to extraction solvents. When applicable, reference antibiotic discs were included as positive controls to validate assay performance and confirm bacterial susceptibility patterns.

Although agar diffusion assays provide valuable preliminary insights into antimicrobial activity, it is recognized that diffusion-based methods are influenced by molecular size, polarity, and agar diffusion characteristics, which may not directly reflect true bacteriostatic potency (Wiegand et al., 2008). Therefore, further quantitative evaluation using broth microdilution assays to determine minimum inhibitory concentration (MIC) is recommended for precise assessment of antimicrobial strength.

2.6 Statistical Analysis

Results were expressed as mean ± SD. One-way ANOVA followed by Tukey's test was performed using SPSS. Differences were considered significant at $p \leq 0.05$.

3. Results

3.1 Extraction Yield

The extraction efficiency of *Retama raetam* aerial parts varied according to solvent polarity (Table 1). Ethanol yielded the highest percentage of extractable material (18.4%), followed by methanol (14.7%), whereas distilled water produced the lowest yield (9.2%).

Table 1. Extraction yield of *Retama raetam* extracts

Solvent	Extraction yield (%)
Ethanol	18.4
Methanol	14.7
Water	9.2

The observed differences indicate that semi-polar solvents were more effective in recovering extractable constituents from the plant matrix compared to highly polar aqueous systems. The higher recovery obtained with ethanol suggests a greater abundance of ethanol-soluble compounds within the tested plant material.

3.2 Qualitative Phytochemical Profile

Qualitative screening revealed the presence of multiple classes of secondary metabolites with variable distribution among solvents (Table 2).

Table 2. Qualitative phytochemical screening

Phytochemical	Aqueous	Methanol	Ethanol
Alkaloids	++	+	+++
Flavonoids	+++	+	+++
Tannins	++	+++	+++
Terpenoids	++	+++	++
Saponins	+++	–	–
Glycosides	–	–	–

Alkaloids were strongly detected in the ethanolic extract (+++), moderately in the aqueous extract (++), and weakly in methanol (+). Flavonoids were highly represented in both aqueous and ethanolic extracts (+++), while their presence in methanol was comparatively lower (+). Tannins exhibited strong reactions in both methanolic and ethanolic extracts (+++), with moderate detection in aqueous extract (++).

Terpenoids showed moderate to strong presence across extracts, with methanol demonstrating the strongest reaction (+++). In contrast, saponins were detected exclusively in the aqueous extract (+++), while being absent in organic solvents. Glycosides were not detected in any extract.

These findings demonstrate solvent-dependent selectivity in extracting different phytochemical classes, indicating differential solubility patterns among secondary metabolites.

3.3 Antibacterial Activity

The ethanolic extract exhibited marked antibacterial activity, with the highest susceptibility observed in Pseudomonas aeruginosa (40 ± 1.2 mm). Moderate inhibition was recorded against Staphylococcus aureus (15 ± 0.5 mm) and Escherichia coli (15 ± 0.7 mm), while Klebsiella spp. showed complete resistance under the tested conditions (Table.3).

Table 3. Antibacterial activity (mean inhibition zone, mm)

Bacterial strain	Ethanolic extract	Amoxicillin
P. aeruginosa	40 ± 1.2	–
S. aureus	15 ± 0.5	15 ± 0.6
E. coli	15 ± 0.7	–
Klebsiella spp.	–	–

The variability in inhibition patterns reflects differential permeability barriers and resistance determinants among tested strains. Gram-negative resistance in Klebsiella spp. may be attributed to capsular polysaccharide protection and β-lactamase-associated defense systems.

4. Discussion

The present findings demonstrate that solvent polarity significantly influenced both extraction yield and antibacterial activity of Retama raetam aerial parts. The higher extraction yield obtained with ethanol compared to methanol and distilled water suggests the predominance of semi-polar bioactive constituents within the plant matrix. Ethanol, as a moderately polar solvent, is capable of dissolving a broad spectrum of phytochemicals, including phenolics, flavonoids, certain alkaloids, and terpenoid derivatives. This observation is consistent with previous reports indicating that ethanol often achieves superior recovery of antimicrobial secondary metabolites compared with highly polar aqueous systems (Azwanida, 2015). The comparatively lower yield in water may reflect limited solubility of lipophilic and semi-polar compounds, thereby restricting extraction efficiency.

Qualitative phytochemical screening confirmed the presence of alkaloids, flavonoids, tannins, terpenoids, and saponins, with a notably stronger representation in the ethanolic extract. Flavonoids and tannins, in particular, are widely documented for their antimicrobial properties, acting through protein precipitation, enzyme inhibition, disruption of membrane permeability, and interference with microbial adhesion (Górniak et al., 2019). Phenolic hydroxyl groups are capable of forming hydrogen bonds with bacterial cell wall proteins and membrane phospholipids, leading to structural destabilization. Additionally, certain flavonoids have been shown to inhibit DNA gyrase and ATP synthase, thereby impairing bacterial replication and energy metabolism. The phytochemical richness observed in the ethanolic extract likely explains its superior antibacterial performance relative to other solvents.

The pronounced inhibitory activity observed against Pseudomonas aeruginosa is particularly noteworthy. This organism is characterized by intrinsic resistance mechanisms, including low outer membrane permeability, efflux pump systems (e.g., MexAB-OprM), and enzymatic degradation pathways (Lister et al., 2009). A 40 mm inhibition zone suggests substantial susceptibility to components present in the crude extract. Although agar diffusion assays may sometimes exaggerate apparent activity due to diffusion characteristics of certain compounds, the

magnitude of inhibition implies possible disruption of membrane integrity or interference with efflux-mediated resistance. Plant-derived phenolic compounds are known to compromise membrane function by increasing permeability and inducing leakage of intracellular contents. Furthermore, synergistic interactions among multiple phytochemicals in crude extracts may enhance antibacterial efficacy beyond that of isolated constituents. Such synergism could potentially inhibit multiple bacterial targets simultaneously, reducing the effectiveness of resistance mechanisms.

In contrast, the absence of detectable inhibition against *Klebsiella spp*. highlights the variability in bacterial susceptibility profiles. The polysaccharide capsule of *Klebsiella pneumoniae* is a well-recognized virulence factor that provides protection against antimicrobial agents and host immune responses (Podschun & Ullmann, 1998). This capsule can limit penetration of bioactive molecules and reduce binding to cellular targets. Additionally, extended-spectrum β-lactamase (ESBL) production and other resistance determinants may further contribute to reduced susceptibility. The differential response observed between *P. aeruginosa* and *Klebsiella spp*. underscores the importance of pathogen-specific evaluation when investigating plant-derived antimicrobials.

Moderate inhibition observed against *Staphylococcus aureus* and *Escherichia coli* aligns with findings reported for other Fabaceae species rich in phenolic and alkaloid compounds. Gram-positive bacteria such as *S. aureus* often exhibit higher sensitivity to plant extracts due to the absence of an outer membrane barrier. However, the comparable inhibition observed against *E. coli* suggests that certain compounds within *R. raetam* may possess sufficient lipophilicity or molecular size to traverse Gram-negative outer membranes. This dual-spectrum activity enhances the pharmacological relevance of the plant.

While agar well diffusion remains a widely used preliminary screening technique, it has inherent limitations. Diffusion-based assays depend on molecular weight, solubility, and diffusion rate of compounds within agar matrices, which may not directly correlate with true antimicrobial potency. Therefore, determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values using broth microdilution methods is recommended to quantify antibacterial strength more precisely. Such quantitative parameters would allow comparison with standard antibiotics and facilitate evaluation of therapeutic potential. Additionally, time-kill assays and membrane integrity studies could provide mechanistic insights into the mode of action.

The current investigation relied on qualitative phytochemical screening, which identifies presence or absence of compound classes but does not quantify their concentrations. Future studies incorporating total phenolic content (TPC), total flavonoid content (TFC), and chromatographic profiling (HPLC or GC–MS) would enhance understanding of the specific compounds responsible for antibacterial activity. Geographic and environmental factors may influence metabolite composition; therefore, seasonal and ecological variability should also be considered in subsequent research.

It is also important to acknowledge that crude extracts represent complex mixtures of bioactive molecules, some of which may act synergistically or antagonistically. Fractionation and bioassay-guided isolation would allow identification of the most potent antibacterial constituents. Moreover, cytotoxicity evaluation against mammalian cell lines is essential to assess safety and therapeutic applicability.

Overall, the observed antibacterial activity of *R. raetam*, particularly against *P. aeruginosa*, supports its potential as a promising source of antimicrobial secondary metabolites. Although preliminary, these findings contribute to the growing evidence that desert-adapted plants may harbor chemically diverse compounds with pharmacological relevance. Further quantitative, mechanistic, and in vivo investigations are warranted to validate and expand upon these results, ultimately bridging the gap between ethnobotanical knowledge and modern antimicrobial drug discovery.

5. Conclusion

The present study confirms that *Retama raetam* ethanolic extract exhibits notable antibacterial activity, likely associated with its rich content of flavonoids, tannins, and alkaloids. The pronounced inhibition against *Pseudomonas aeruginosa* highlights the plant's potential effectiveness against resistant Gram-negative pathogens. Differences in bacterial susceptibility emphasize the selective action of its bioactive constituents. Although based on preliminary qualitative and diffusion assays, these findings support *R. raetam* as a promising source of antimicrobial secondary metabolites. Further quantitative analyses, MIC determination, and mechanistic studies are necessary to validate its therapeutic relevance and advance its potential application in antimicrobial drug development.

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